

## ORIGINAL ARTICLE

# Correlates of plasma and platelet tissue factor pathway inhibitor, factor V, and Protein S

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## Summary

**Background:** Plasma Tissue Factor Pathway Inhibitor (TFPI) circulates bound to factor V (fV) and Protein S (PS). Estrogen therapy decreases plasma TFPI and PS. TFPI, fV, and PS circulate within platelets, and are released upon activation to modulate thrombus formation.

**Objective:** Identify factors affecting the concentrations of plasma and platelet TFPI, fV, and PS.

**Methods:** Blood samples were obtained from 435 healthy individuals. Plasma total TFPI, TFPI $\alpha$ , fV, and PS, and platelet TFPI, fV, and PS were quantified. Correlations between these protein concentrations and age, gender, race, and estrogen use were established.

**Results:** In males, only plasma fV increased with age, while in females, all plasma analytes increased with age. Males had higher plasma total TFPI, TFPI $\alpha$ , and PS than females. The platelet proteins in either sex remained relatively stable with increasing age. Platelet TFPI and PS were comparable in both sexes, while platelet fV was higher in females. Estrogen use was associated with decreased plasma total TFPI and TFPI $\alpha$ , and platelet PS, but not with platelet TFPI concentration. Racial differences in plasma and platelet proteins were observed, some of which were larger than inter-individual differences observed within racial groups. TFPI, fV and PS concentrations correlated in plasma, while only fV and PS correlated in platelets.

**Conclusions:** Plasma and platelet TFPI, fV and PS differ in their: (i) *in vivo* association; (ii) demographic correlates; and (iii) alteration by estrogen therapies. Therefore, the plasma and platelet pools of these proteins may modulate hemostasis and thrombosis via different biochemical pathways.

## KEYWORDS

factor V, hemophilia, platelets, protein, TFPI

## Essentials

- Tissue Factor Pathway Inhibitor, Protein S, and Factor V circulate in plasma and platelet pools.
- These proteins were measured in plasma and platelets from 435 healthy subjects and correlated.
- Plasma and platelet pools differ in their demographic correlates and alterations by estrogen.
- Plasma and platelet pools may differentially modulate thrombotic and hemostatic responses.

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## 1 | INTRODUCTION

Tissue factor pathway inhibitor (TFPI) is an anticoagulant protein. It modulates the extrinsic coagulation pathway through factor Xa (fXa) dependent inhibition of the tissue factor (TF)-factor VIIa (fVIIa) complex and the common coagulation pathway through inhibition of early forms of prothrombinase. Alternative splicing gives rise to two TFPI isoforms, TFPI $\alpha$  and TFPI $\beta$ . Both isoforms directly inhibit fXa and mediate fXa-dependent inhibition of TF-fVIIa. TFPI $\alpha$ , but not TFPI $\beta$ , inhibits prothrombinase via its association with forms of activated factor V (fVa) that retain an acidic region within its B-domain (for a review, see Wood et al<sup>1</sup>).

Mice lacking TFPI die in utero from a consumptive coagulopathy,<sup>2</sup> and a TFPI deficient human has not been described, suggesting that TFPI is required for embryonic survival in humans. Patients producing the East Texas and Amsterdam variants of fV,<sup>3,4</sup> both of which tightly associate with plasma TFPI $\alpha$ , highlight the clinical importance of TFPI. These patients have 10- to 20-fold increased plasma TFPI $\alpha$ , an amount sufficient to cause a moderate bleeding diathesis. In vitro and in vivo studies,<sup>5,6</sup> including in a recent Phase 1b clinical trial,<sup>7</sup> have found that inhibition of TFPI using anti-TFPI antibodies may be efficacious for the treatment of hemophilia, further exemplifying its clinical importance.

Circulating TFPI is present in plasma<sup>8</sup> and platelets.<sup>9,10</sup> The majority of plasma TFPI is a C-terminally truncated form bound to lipoproteins.<sup>11</sup> Lipoprotein-associated TFPI has reduced anticoagulant activity.<sup>12</sup> However, it is capable of limiting thrombin generation during the propagation phase of coagulation.<sup>13</sup> The remainder of plasma TFPI is TFPI $\alpha$ , also termed full-length TFPI or free TFPI.<sup>11</sup> Protein S (PS) is a co-factor that enhances the inhibition of fXa by TFPI $\alpha$ .<sup>14</sup> TFPI $\alpha$  circulates bound to PS<sup>15</sup> or to fV.<sup>16</sup> Consequently, PS or fV deficiency is associated with decreased plasma TFPI $\alpha$ .<sup>15,16</sup> Plasma TFPI antigen and activity are also altered by estrogen, decreasing 15-20% in women using oral contraceptives or estrogen replacement therapy.<sup>17-20</sup>

Approximately 10% of circulating TFPI is concentrated within quiescent platelets<sup>9,10</sup> and is entirely TFPI $\alpha$ .<sup>10,21</sup> TFPI $\alpha$  is secreted from activated platelets,<sup>21</sup> with a portion also localizing to the activated platelet surface.<sup>10</sup> Interestingly, a lack of thrombin-mediated platelet activation successfully rescues the embryonic lethal phenotype of mice lacking TFPI,<sup>22</sup> suggesting an important link between platelet TFPI and thrombin-mediated platelet activation. Furthermore, mouse models have revealed that platelet TFPI modulates clot formation after electrolytic blood vessel injury<sup>23</sup> and bleeding severity in hemophilic mice.<sup>24</sup> These murine experiments, along with the biochemical data uncovering the inhibition of prothrombinase by TFPI $\alpha$ ,<sup>25</sup> have implicated the inhibition of platelet TFPI anticoagulant activity as a potential therapeutic target for haemophilia.<sup>26</sup>

Determining how plasma and platelet TFPI, fV, and PS vary by demographic characteristics and estrogen use, and the in vivo association of these proteins, is necessary for understanding how alterations in plasma or platelet TFPI may relate to the severity of bleeding and clotting disorders. Therefore, assays for the measurement of plasma total TFPI, plasma TFPI $\alpha$ , and platelet TFPI were developed using high affinity monoclonal antibodies. These assays were used to quantify

the different circulating TFPI pools with comparison to fV and PS in 435 healthy individuals.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics approval

The Institutional Review Board of the Blood Center of Wisconsin approved the use of human subjects for this study. Informed, written consent for study participation was obtained from all subjects before sample collection.

### 2.2 | Blood collection and processing

Blood was collected into 3.2% sodium citrate and centrifuged (150  $\times$  g, 15'). The platelet-rich plasma (PRP) was collected and an equal volume of BSGC buffer (129 mmol L<sup>-1</sup> NaCl, 13.6 mmol L<sup>-1</sup> Na<sub>3</sub>Citrate, 11.1 mmol L<sup>-1</sup> Glucose, 1.6 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 8.6 mmol L<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>, 100 ng mL<sup>-1</sup> prostaglandin E-1 [PGE-1], pH 7.3) added. Platelets were pelleted by centrifugation (650  $\times$  g, 10'), washed twice with BSGC buffer, counted, and lysed (20 mmol L<sup>-1</sup> Tris-HCl, 150 mmol L<sup>-1</sup> NaCl, 1 mmol L<sup>-1</sup> Na<sub>2</sub>EDTA, 1 mmol L<sup>-1</sup> EGTA, 1% Triton X-100, 1  $\mu$ g mL<sup>-1</sup> E-64 [N-(N-[L-3-trans-carboxyirane-2-carbonyl]-L-leucyl)-agmatine], 1 mmol L<sup>-1</sup> phenylmethanesulfonyl fluoride [PMSF], pH 7.5) at a concentration of 1  $\times$  10<sup>9</sup> platelets L<sup>-1</sup>. The lysate was clarified by centrifugation (14 000  $\times$  g, 10', 4°C). For plasma isolation, the blood sample remaining after removal of PRP was centrifuged (2500  $\times$  g, 20') and the plasma collected. Plasmas and platelet lysates were stored at -80°C until assay. TFPI $\alpha$ , fV, PS, and total protein content of each lysate were determined, and the latter used to standardize the final platelet TFPI $\alpha$ , fV and PS concentrations reported.

### 2.3 | Total TFPI and TFPI $\alpha$ bead-based proximity assays

All antibodies used were mouse monoclonal antibodies directed against different regions of human TFPI. An antibody against the second Kunitz domain ( $\alpha$ -K2) was conjugated to acceptor beads (AlphaLISA and AlphaSCREEN beads, Perkin Elmer, Waltham, MA, USA). Both total TFPI and TFPI $\alpha$  assays used these beads. The total TFPI assay used antibody against the first Kunitz domain (a gift from Dr George Broze Jr., Washington University, St Louis, MO, USA). This antibody was biotinylated ( $\beta$  $\alpha$ -K1) and used to detect all forms of TFPI, while the TFPI $\alpha$  assay used a biotinylated antibody against the third Kunitz domain ( $\beta$  $\alpha$ -K3). A streptavidin-conjugated donor bead (SA) was used to detect the biotinylated antibody. AlphaLISA acceptor beads were used for measurement of plasma TFPI. AlphaSCREEN acceptor beads were used for measurement of platelet TFPI. To perform the assay, 5  $\mu$ L of sample (1/20 dilution of plasma or platelet lysate, diluted in 50 mmol L<sup>-1</sup> HEPES, 100 mmol L<sup>-1</sup> NaCl, 0.1% bovine serum albumin, 0.1% Tween-20, pH 7.5, containing 400  $\mu$ g mL<sup>-1</sup> HBR-1 heterophile antibody blocking reagent [Scantibodies, Santee, CA, USA]) and 20  $\mu$ L of a mixture containing 12.5  $\mu$ g mL<sup>-1</sup>  $\alpha$ -K2 conjugated acceptor bead and 2.5 nmol L<sup>-1</sup>  $\beta$  $\alpha$ -K1

or 5 nmol L<sup>-1</sup> b $\alpha$ -K3 was added to the well of a 96-well plate and incubated (1 h, 37°C). SA was added (25  $\mu$ L, 20  $\mu$ g mL<sup>-1</sup>) and incubated (1 h, RT). The plate was read on Enspire plate reader using the AlphaLISA program (Perkin Elmer, Waltham, MA, USA), and the sample results obtained by interpolation from a standard curve of glycosylated recombinant human TFPI $\alpha$  produced in BHK cells, with a dynamic range between 0.1 and 10 ng mL<sup>-1</sup> for the total TFPI assay and 0.04-2.5 ng mL<sup>-1</sup> for the TFPI $\alpha$  assay. The lower limit of quantification (LLOQ) in all assays was 0.05 ng mL<sup>-1</sup>, corresponding to the lowest calibrator point. With a fixed sample dilution of 1:20, sample LLOQ was 1 ng mL<sup>-1</sup>.

## 2.4 | Measurement of total and free TFPI, and factor V and protein S

Total and free TFPI (Stago UK, England) and fV and total PS (Enzyme Research Laboratories, South Bend, IN, USA) ELISAs were performed according to manufacturers' instructions.

## 2.5 | Statistical analysis

All analytes were analyzed separately by the following method. Data were log transformed (natural logarithm) prior to statistical analysis, and differences on the log scale were back-transformed (by the exp function) to the original scale as ratios (or fold). Data were analysed by a series of models. The initial model included the following main factors as explanatory variables: Age at enrollment as a continuous regression parameter in all models; Race/Ethnicity (Asian, African American, Caucasian); Gender (male, female); Use of oral contraceptives or estrogen (yes, no); ABO Group (ABNeg, ABPos, ANeg, APos, BNeg, BPos, ONeg, OPos). The initial model further included those interaction terms, which were identifiable from data. The initial model was reduced by removal of non-significant terms (respecting the model hierarchy). ABO Group had no effect on any measured analyte and, therefore, was removed from all models. Equations used to calculate adjusted values are shown in Table S1. The correlation between the seven parameters was estimated by the restricted maximum likelihood method using the log-transformed results. Calculations were performed using SAS JMP version 12.0.1.

# 3 | RESULTS

## 3.1 | Study populations

A total of 435 healthy subjects provided informed consent to participate in this study, ranging in age from 18 to over 70 (Table 1). Most were Caucasian, however, 114 were African American, and 25 were Asian by self-report. Some participants were routine whole blood donors, who provided an additional 5 mL blood sample collected from the pre-donation pouch. Others provided the blood sample via phlebotomy performed specifically for this study. Samples were collected in the morning (0800-1200) and donors were not directed to fast before collection. All participants provided a list of current medications. Females listed specific use of estrogen containing drugs, and none were pregnant.

**TABLE 1** Demographic characteristics of the study population

Demographic Characteristic	Number of Donors	
Gender		
Male	215	
Female	220	
Oral Contraceptive Use (OC)	39	
Estrogen Therapy Use (ET)	6	
Neither OC or ET	175	
Age (years)	Male	Female
< 30	57	41
30-39	33	45
40-49	31	48
50-59	44	44
60-69	35	26
70+	15	16
Race/ethnicity	Male	Female
Caucasian	169	121
African American	27	87
Asian	16	9
Unknown	3	4

## 3.2 | Validation of TFPI antigen assays

Bead-based proximity assays were developed in our laboratory to measure total TFPI and TFPI $\alpha$  antigen in plasma and platelets using a well-characterized anti-K1 monoclonal antibody<sup>11</sup> and high affinity (Kd  $\leq$  25 pM) anti-K2,<sup>6</sup> and anti-K3<sup>27</sup> TFPI monoclonal antibodies. All TFPI assays were calibrated using recombinant glycosylated human TFPI produced in BHK cells. In each assay, the dose response was linear for the TFPI range tested and the inter-assay CV at each concentration was <20% (Fig. S1 and Table S2). Intra- and inter-assay reproducibility was determined using a pool of plasma or platelets from 20 male blood donors and ranged from 13.2 to 15.4% (Table S3). Spike recovery of 5, 10, and 50 ng mL<sup>-1</sup> TFPI in pooled plasma showed recovery between 96% and 118%, indicating acceptable accuracy in both assays. The total TFPI and TFPI $\alpha$  concentrations in TFPI-immunodepleted plasma were less than the LLOQ, demonstrating that the assays specifically measure TFPI. Comparison of the in-house total TFPI and TFPI $\alpha$  assays and commercially available ELISA kits was performed using seven different male plasma samples. Except for a single outlier, mean total TFPI and TFPI $\alpha$  values determined by each assay were comparable (Table S4).

## 3.3 | Measurement of plasma TFPI, fV, and PS in males

Male and female data were analyzed separately because of the well-established association between estrogen use and decreased plasma TFPI in females.<sup>17</sup> Plasma total TFPI, TFPI $\alpha$ , fV, and PS were measured in samples obtained from 215 healthy males (Table 2). The average total TFPI and TFPI $\alpha$  were 62.2 and 14.7 ng mL<sup>-1</sup>, respectively. Therefore,

TFPI $\alpha$  represented 23.6% of the total plasma TFPI in males. The average fV and PS were 21.1 and 30.0  $\mu\text{g mL}^{-1}$ , respectively. Male subjects were stratified by age to examine for changes in plasma TFPI, fV and PS. Plasma fV increased by 0.29% per year ( $P = .0003$ ), which is consistent with reports suggesting that plasma fV increases with age.<sup>28</sup> There were no changes with age in plasma total TFPI, TFPI $\alpha$ , or PS.

### 3.4 | Measurement of plasma TFPI, fV, and PS in females

Total TFPI, TFPI $\alpha$ , fV, and PS were measured in plasma samples from 220 female blood donors (Table 2). The average total TFPI

and TFPI $\alpha$  were 54.3 and 11.3  $\text{ng mL}^{-1}$ , respectively. Therefore, TFPI $\alpha$  represented 20.8% of the total plasma TFPI in females. The average fV and PS were 22.0 and 25.1  $\mu\text{g mL}^{-1}$ , respectively. Female subjects were stratified by age to examine for changes in plasma TFPI, fV and PS. Female fV levels increased by 0.29% per year ( $P = .0003$ ), identical to that observed for male subjects. In contrast to that observed in male subjects, age associated increases in plasma TFPI and PS were observed in female subjects. Plasma total TFPI increased by 0.55% per year ( $P = .0006$ ). Plasma TFPI $\alpha$  increased by 1.13% per year ( $P = 5 \times 10^{-12}$ ). Plasma PS increased by 0.85% per year ( $P = 4e-7$ ). Comparison of values from male and female subjects

**TABLE 2** Mean plasma concentration of total TFPI, TFPI $\alpha$ , fV and PS

	Total TFPI (ng/mL) <sup>†</sup>		TFPI $\alpha$ (ng/mL) <sup>‡, §, ¶</sup>	
	Male	Female*	Male	Female*
Total	62.2 (59.6-64.7; 215)	54.3 (51.4-57.2; 220)	14.7 (14.1-15.2; 215)	11.3 (10.7-12.0; 220)
Age				
<30	66.6 (60.3-73.0; 57)	48.8 (40.7-57.0; 41)	14.5 (13.5-15.4; 57)	8.6 (7.5-9.6; 41)
30-39	60.0 (52.8-67.2; 33)	50.6 (44.5-56.7; 45)	13.4 (11.8-15.1; 33)	8.7 (7.7-9.6; 45)
40-49	60.4 (54.7-66.0; 31)	50.3 (45.3-55.4; 48)	14.3 (12.8-15.8; 31)	10.9 (9.4-12.5; 48)
50-59	56.0 (51.0-61.1; 44)	61.8 (54.4-69.1; 44)	13.8 (12.8-14.9; 44)	13.2 (11.8-14.6; 44)
60-69	64.2 (58.8-69.6; 35)	56.3 (49.7-62.7; 26)	16.4 (15.2-17.6; 35)	13.4 (12.0-14.8; 26)
>70	66.8 (57.8-75.8; 15)	67.3 (55.4-79.1; 16)	17.3 (14.9-19.8; 15)	18.8 (15.3-22.3; 16)
Race/ethnicity				
Caucasian	63.0 (60.2-65.8; 169)	57.8 (53.4-62.2; 121)	15.3 (14.8-15.9; 169)	12.5 (11.4-13.5; 121)
African American	58.1 (49.1-67.1; 27)	50.1 (46.1-54.1; 87)	12.4 (10.9-14.0; 27)	9.9 (8.9-10.8; 87)
Asian	59.9 (48.2-71.6; 16)	50.6 (39.7-61.5; 9)	11.5 (9.6-13.5; 16)	9.5 (7.7-11.2; 9)
	Factor V ( $\mu\text{g/mL}$ )		Protein S ( $\mu\text{g/mL}$ ) <sup>‡, §, **, ††</sup>	
	Male*	Female*	Male	Female*
Total	21.1 (20.3-21.9; 215)	22.0 (21.2-22.8; 219)	30.0 (28.6-31.4; 213)	25.1 (23.7-26.4; 216)
Age				
<30	20.0 (18.9-21.1; 57)	21.2 (19.5-22.9; 40)	31.7 (29.3-34.2; 57)	21.3 (19.0-23.6; 39)
30-39	18.9 (16.5-21.2; 33)	21.5 (19.8-23.2; 45)	28.6 (24.9-32.4; 33)	21.5 (19.3-23.8; 44)
40-49	20.9 (18.4-23.3; 31)	20.7 (19.2-22.3; 48)	25.4 (22.3-28.4; 31)	25.1 (21.8-28.4; 48)
50-59	21.5 (19.6-23.3; 44)	23.2 (21.5-25.0; 44)	28.7 (25.5-31.9; 43)	26.9 (23.8-30.0; 44)
60-69	24.4 (22.4-26.4; 35)	24.6 (22.1-27.1; 26)	33.9 (30.3-37.5; 34)	27.5 (23.9-31.2; 25)
>70	22.5 (19.3-25.6; 15)	21.4 (18.7-24.1; 16)	30.8 (24.9-36.7; 15)	35.3 (29.1-41.6; 16)
Race/ethnicity				
Caucasian	21.1 (20.2-22.0; 169)	21.8 (20.8-22.8; 120)	31.7 (30.2-33.2; 167)	28.0 (26.0-30.1; 119)
African American	22.3 (19.9-24.8; 27)	22.4 (21.0-23.7; 87)	25.4 (22.5-28.2; 27)	22.3 (20.9-23.7; 86)
Asian	18.7 (16.3-21.0; 16)	22.0 (19.1-25.0; 9)	20.4 (15.6-25.1; 16)	15.0 (9.4-20.7; 8)

The numbers in parentheses represent the 95% confidence interval and the number of donors in that group.

\* $P < .001$  for difference with increasing age.

† $P < .05$  for difference between sexes.

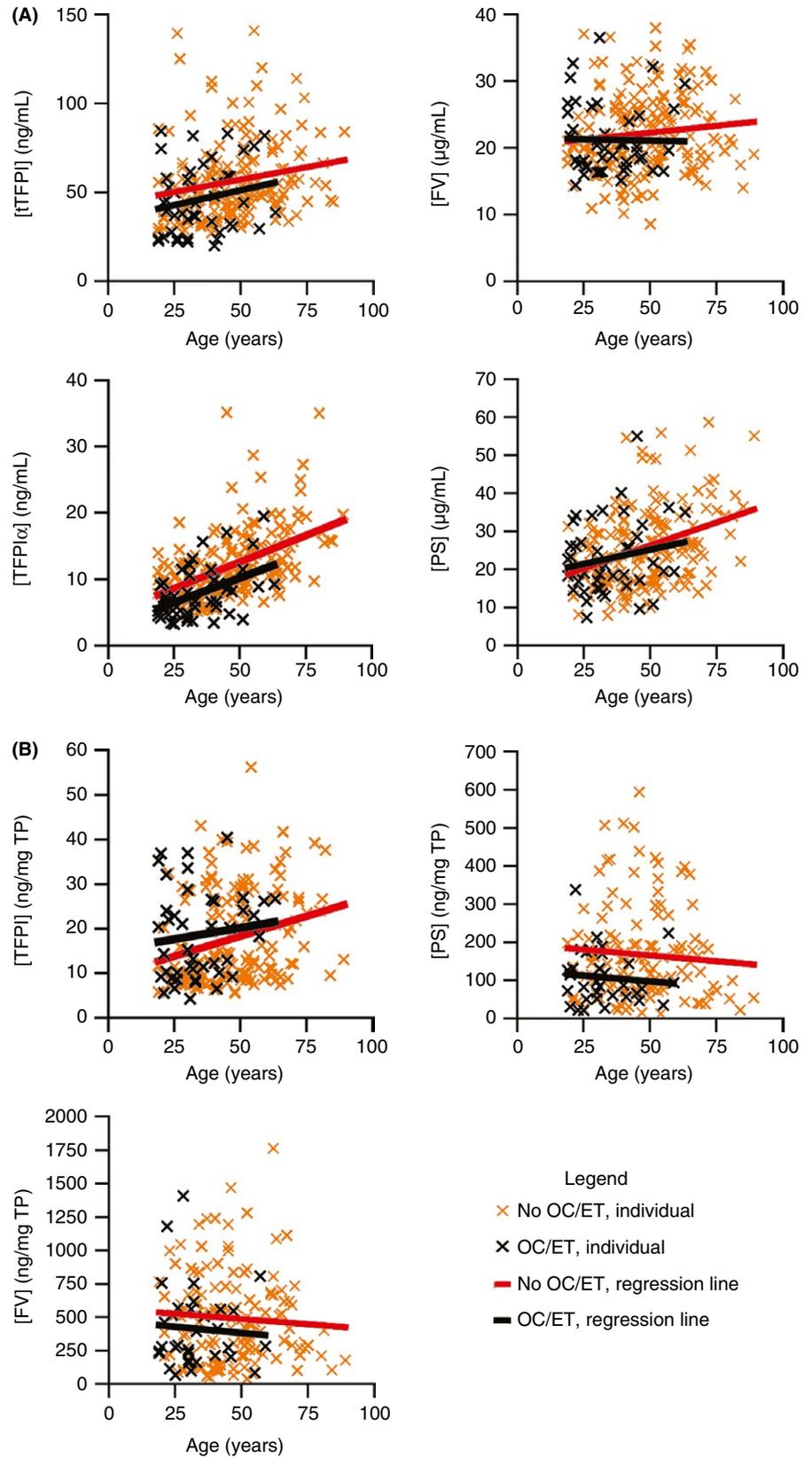
‡ $P < .0001$  for difference between sexes.

§ $P < .0001$  for difference between Caucasian and African American.

¶ $P < .05$  for difference between Caucasian and Asian.

\*\* $P < .0001$  for difference between Caucasian and Asian.

†† $P < .0001$  for difference between African American and Asian.



**FIGURE 1** Association of estrogen therapies with (A) Plasma Total TFPI, TFPI $\alpha$ , fV, and PS and (B) Platelet TFPI, fV and PS in females. Individual values for donors not on OC or ET therapy are represented by an orange x (x), and those on OC or ET therapy by a black x (x). Regression lines for donors either not on OC/ET therapy or on OC/ET therapy are shown in red or black, respectively. TP, Total Protein

revealed that, after adjusting for age, race and oral contraceptive/estrogen use, males have 8.8% higher plasma TFPI $\alpha$  ( $P < .0001$ ), 4.3% higher plasma total TFPI ( $P = .01$ ), and 7.7% higher plasma PS ( $P < .0001$ ) than females, while males and females have equivalent plasma fV.

### 3.5 | Association between oral contraceptive/estrogen use and plasma TFPI, fV, and PS

Six females reported use of estrogen replacement therapy and 39 reported use of oral contraceptives (Table 1). When combined as a

single group, female subjects on these two therapies had 25.2% lower plasma TFPI $\alpha$  (Figure 1A;  $P < .001$ ) and 19.1% lower plasma total TFPI (Figure 1A;  $P = .005$ ) than females not using these medications. There was no significant difference between the two groups in plasma fV or PS concentration (Figure 1A).

### 3.6 | Measurement of platelet TFPI, fV, and PS in males

TFPI $\alpha$  is stored within quiescent platelets but is not localized within  $\alpha$ -granules.<sup>9,10</sup> fV<sup>29</sup> and PS<sup>30</sup> are localized within  $\alpha$ -granules of quiescent platelets. The intra-cellular concentrations of all three proteins were measured in quiescent platelet lysates obtained from 215 male blood donors (Table 3). The average TFPI $\alpha$ , fV, and PS were 20.7, 343.9, and 159.0 ng mg<sup>-1</sup> total protein, respectively. Statistical analysis revealed that platelet TFPI increases by 0.31% per year ( $P = .0423$ ) in males, while platelet fV and PS do not vary with age.

### 3.7 | Measurement of platelet TFPI, fV and PS in females

Platelet TFPI, fV, and PS were determined in samples obtained from the 220 healthy females (Table 3). The average TFPI, fV, and PS were 17.9, 482.2, and 157.9 ng mg<sup>-1</sup> total protein, respectively. Statistical analysis revealed that platelet TFPI also increases by 0.31% per year ( $P = .0423$ ) in females, while platelet fV and PS do not vary with age. Comparison of values from male and female subjects demonstrated that, on average, females have 9% higher platelet fV than males ( $P = .04$ ) with no differences in platelet TFPI or PS present.

### 3.8 | Association between oral contraceptive/estrogen use and platelet TFPI, fV, and PS

Female subjects reporting use of oral contraceptives or estrogen replacement therapy had, on average, 29.5% lower platelet PS concentration ( $P = .01$ ) than females not using these medications (Figure 1B). These therapies were not associated with platelet TFPI or fV concentration (Figure 1B).

### 3.9 | Variation in plasma and platelet TFPI, fV, and PS between races/ethnicities

Examination of race/ethnicity revealed differences in TFPI, fV, and PS concentration in plasma and platelets (Tables 2 and 3). On average, plasma TFPI $\alpha$  was 19.3% higher in Caucasians than African Americans ( $P < .0001$ ) and 27.4% higher in Caucasians than Asians ( $P < 0.01$ ). Similarly, plasma PS was 17.8% higher in Caucasians than African Americans ( $P < .001$ ) and 80.3% higher in Caucasians than Asians ( $P < .0001$ ). Accordingly, plasma PS was 53.0% higher in African Americans than Asians ( $P < .0001$ ). There were no racial differences in plasma total TFPI or plasma fV. Caucasians have, on average, much higher platelet TFPI than African Americans (131.9%) and Asians (117.3%) ( $P < .0001$  for both) and have much lower platelet fV

(-48.2% vs. African Americans,  $P < .0001$ ; -45.3% vs. Asians,  $P < .01$ ) and platelet PS (-40.4% vs. African Americans,  $P < .0001$ ; -43.8% vs. Asians,  $P < .01$ ). Interestingly, platelet fV increases approximately 2% per year in African Americans ( $P = .0005$ ) but not in other races/ethnicities.

### 3.10 | Inter-individual variation of TFPI, fV, and PS in plasma and platelets

The inter-individual variation of the concentrations of TFPI, fV, and PS within plasma and platelets was evaluated after stratifying by gender and race (Figure 2). The TFPI concentration in plasma and platelets varied by 5- to 10-fold between individuals, while the fV and PS concentrations varied by 5- to 40-fold, with even greater variance observed in platelets. To determine if the effect of inter-individual variation was greater than that of race/ethnicity, the root mean square error, as an estimate of the relative standard deviation (and therefore variation) within the population was determined for each protein measured (Table 4). The estimated inter-individual variation for plasma total TFPI, TFPI $\alpha$ , fV, and platelet fV and PS was either approximately equal to or greater than the difference observed between racial groups, demonstrating that inter-individual variation has a greater effect than racial/ethnic variation on the concentration of these proteins. However, for plasma protein S, the difference between the Asian cohort and both Caucasians and African Americans was greater than the inter-individual variation. Similarly, for platelet TFPI, the difference between Caucasians and both African Americans and Asians was greater than the inter-individual variation.

### 3.11 | Correlation of TFPI, fV, and PS in plasma and platelets

Correlations between TFPI, fV and PS values in plasma and platelets were established (Figure 3 and Table S5). It has been reported that plasma TFPI $\alpha$  correlates with the plasma concentrations of fV<sup>16</sup> and PS,<sup>15</sup> with evidence that TFPI circulates in plasma bound to either fV or PS. Consistent with these previous findings, the present data found a weak correlation between plasma TFPI $\alpha$  and fV ( $r^2 = .18$ ) and a moderate correlation between plasma TFPI $\alpha$  and PS ( $r^2 = .40$ ). In addition, a weak correlation between plasma fV and plasma PS was also present ( $r^2 = .16$ ). Examination of platelet data revealed a strong correlation between platelet fV and platelet PS ( $r^2 = .58$ ), which is consistent with the localization of these two proteins within the platelet  $\alpha$ -granule. However, negative correlations were observed between platelet TFPI and platelet fV ( $r^2 = -0.23$ ) or platelet PS ( $r^2 = -.22$ ), which is consistent with TFPI $\alpha$  not being localized within platelet  $\alpha$ -granules.

## 4 | DISCUSSION

TFPI, fV, and PS are present in plasma and in platelets. Previous studies have characterized the interactions of these proteins within

**TABLE 3** Mean Platelet Concentrations of TFP1 $\alpha$ , FV, and PS

	TFP1 $\alpha$ (ng/mg protein) <sup>†, §</sup>		Factor V (ng/mg protein) <sup>†, ‡, ¶, ¶¶</sup>		Protein S (ng/mg protein) <sup>†, ¶¶</sup>	
	Male*	Female*	Male	Female	Male	Female
Total	20.7 (18.9-22.4; 190)	17.9 (32.9-38.6; 202)	343.9 (301.4-386.5; 158)	482.2 (432.1-532.3; 170)	159.0 (139.9-178.2; 158)	157.9 (140.2-175.6; 168)
Age						
<30	21.0 (17.8-24.2; 51)	15.2 (12.3-18.1; 39)	289.5 (218.9-360.1; 44)	485.9 (364.3-607.5; 32)	112.5 (88.8-136.1; 44)	129.3 (101.0-157.6; 31)
30-39	16.4 (12.4-20.4; 31)	16.7 (13.6-19.9; 45)	474.4 (332.2-616.6; 27)	484.4 (378.2-590.7; 40)	221.9 (148.6-295.1; 27)	168.3 (129.9-206.8; 40)
40-49	16.7 (13.2-20.2; 31)	16.8 (13.9-19.7; 44)	343.1 (226.6-459.6; 27)	515.6 (399.5-631.8; 38)	160.8 (123.4-198.3; 27)	169.2 (121.6-216.9; 38)
50-59	22.8 (17.6-28.0; 35)	20.1 (16.5-23.7; 39)	301.7 (227.1-376.4; 26)	397.5 (304.9-490.0; 33)	190.9 (146.8-235.0; 26)	165.7 (125.4-206.1; 32)
60-69	25.0 (20.7-29.3; 30)	20.3 (15.7-25.0; 23)	354.2 (242.6-465.8; 23)	680.7 (490.2-871.3; 17)	153.3 (101.2-205.4; 23)	187.1 (127.7-246.6; 17)
>70	23.3 (17.2-29.3; 12)	23.1 (17.1-29.0; 12)	321.8 (158.3-485.3; 11)	276.4 (145.0-407.9; 10)	122.9 (60.8-185.0; 11)	86.2 (44.7-127.7; 10)
Race/ethnicity						
Caucasian	23.7 (21.8-25.7; 144)	25.0 (23.2-26.8; 106)	242.5 (212.2-272.8; 113)	343.1 (272.0-414.3; 75)	137.9 (115.9-160; 113)	101.2 (82.6-119.8; 73)
African American	9.9 (8.4-11.5; 27)	10.1 (9.4-10.7; 84)	536.3 (419.5-653.0; 27)	571.6 (510.5-632.7; 84)	194.6 (162.9-226.2; 27)	199.0 (173.7-224.3; 84)
Asian	11.6 (8.5-14.7; 16)	10.3 (8.7-11.9; 9)	732.5 (555.4-909.6; 16)	679.3 (324.6-1034.0; 8)	254.4 (171.9-337.0; 16)	215.1 (130.1-300.0; 8)

The numbers in parentheses represent the 95% confidence interval and the number of donors in that group.

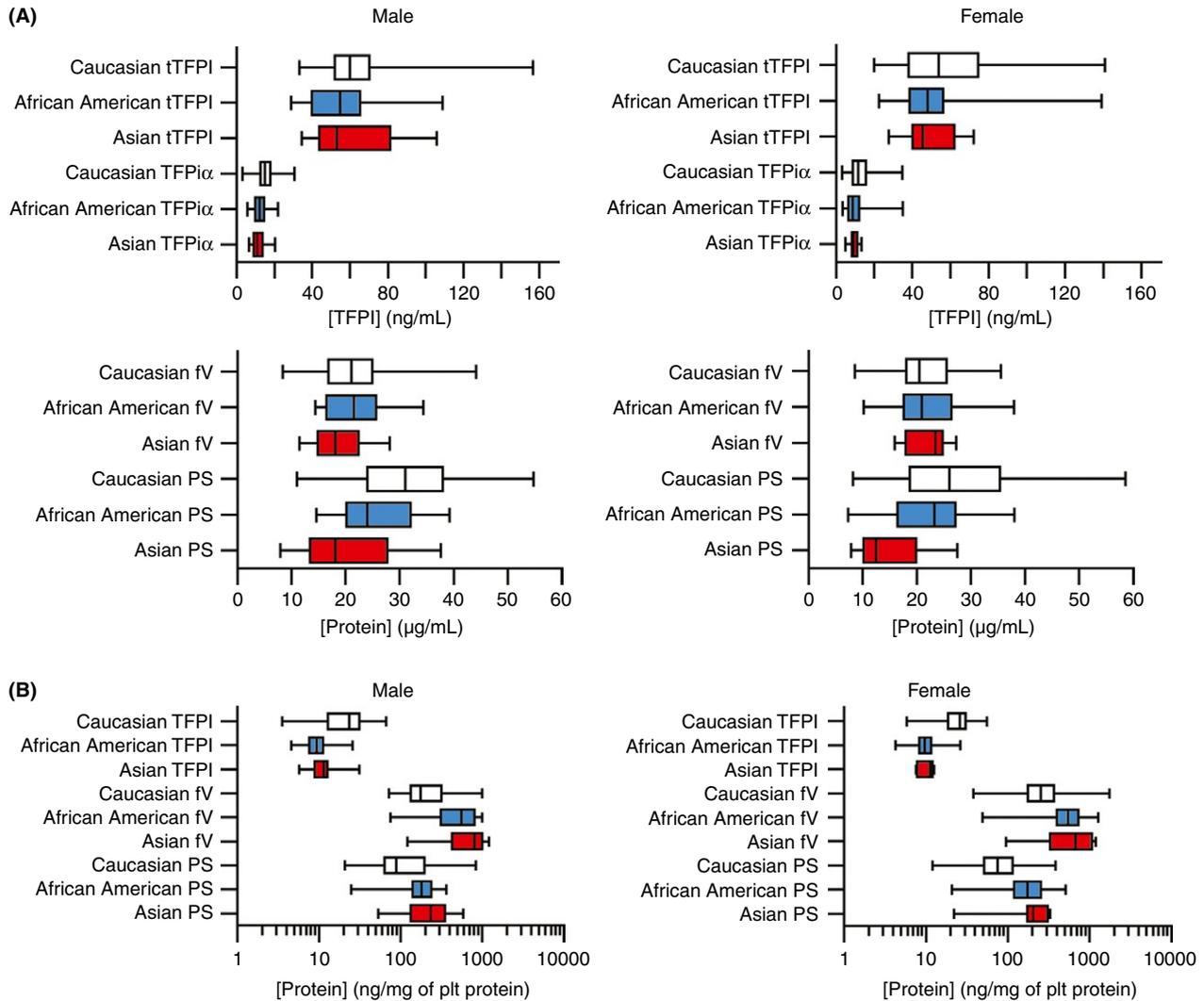
\* P < .05 for difference with increasing age.

† P < .05 for difference between sexes.

‡ P < .001 for difference between Caucasian and African American.

§ P < .001 for difference between Caucasian and Asian.

¶ P < .05 for difference between Caucasian and Asian.



**FIGURE 2** Inter-individual variability of (A) Plasma Total TFPI, TFPI $\alpha$ , fV, and PS and (B) Platelet TFPI, fV, and PS. In individual box-and-whiskers of each plot, the outside lines of the box represent the 25th and 75th percentiles, and the middle line the median value. The whiskers represent the minimum and maximum value

plasma, concluding that TFPI circulates in complex with either fV<sup>16</sup> or with PS,<sup>15</sup> but not in a ternary complex with both.<sup>15</sup> These interactions alter the biological activity of TFPI by enhancing its anticoagulant activity and prolonging its circulatory half-life. Murine studies have found that the platelet pools of these three proteins exert biological activity in thrombosis and hemostasis models.<sup>23,24,31-34</sup> Here, we measured platelet and plasma TFPI, fV, and PS in 435 healthy subjects in order to study demographic parameters associated with changes in protein concentrations and identify in vivo correlates between their plasma and platelets pools. These data defined and quantified the sex-, racial-, and age-related differences in plasma and platelet TFPI, fV, and PS concentrations, finding they distinctly differ in regards to their in vivo association, their demographic correlates and their alteration by estrogen therapies.

Bead-based proximity assays developed for these studies found the average plasma total TFPI and TFPI $\alpha$  concentrations to be approximately 60 and 12.5 ng mL<sup>-1</sup>, respectively, such that TFPI $\alpha$  accounted

for approximately 20% of total TFPI in plasma. These values were consistent with those obtained using commercially available TFPI ELISA assays, validating the use of the new assays for the measurement of total TFPI and TFPI $\alpha$ . fV and PS were measured using commercially available ELISA assays. The plasma TFPI $\alpha$  concentration has been reported to correlate with the plasma concentration of fV<sup>16</sup> and of PS.<sup>15</sup> A correlation between plasma TFPI $\alpha$  and fV was present in the current study ( $r^2 = .18$ ), but was weaker than that reported by Dahm and co-workers ( $r^2 = .39$ )<sup>35</sup> or Duckers and co-workers ( $r^2 = .739$ ).<sup>16</sup> The differences between the latter and the current study may be attributed to the differences in the populations studied. The Duckers study population included healthy and severely fV-deficient individuals providing a large range of plasma protein concentrations for comparison, whereas the current study included healthy individuals only. A relatively strong correlation between plasma TFPI $\alpha$  and PS ( $r^2 = .40$ ) was present in the current study, which is comparable with previous reports.<sup>15</sup>

**TABLE 4** Comparison of the difference in plasma and platelet TFPI, FV, and PS between racial group vs. the estimated inter-individual variation in the study population

	Plasma analytes				Platelet analytes		
	Total TFPI	TFPI $\alpha$	FV	PS	TFPI $\alpha$	FV	PS
Caucasian vs.							
African American	9.1%	19.3% <sup>‡</sup>	-6.7%	17.8% <sup>†</sup>	<b>131.9%</b> <sup>‡</sup>	-48.2% <sup>‡</sup>	-40.4% <sup>‡</sup>
Asian	17.4%	27.4% <sup>*</sup>	5.1%	<b>80.3%</b> <sup>‡</sup>	<b>117.3%</b> <sup>‡</sup>	-45.3% <sup>*</sup>	-43.8% <sup>*</sup>
African American vs.							
Asian	7.6%	6.8%	12.7%	<b>53.0%</b> <sup>‡</sup>	-6.3%	5.6%	-5.7%
Estimated inter-individual variation	<b>32.0%</b>	<b>31.9%</b>	<b>27.3%</b>	34.3%	43.4%	<b>67.1%</b>	<b>68.2%</b>

Shown are the differences between racial/ethnic groups in the mean concentration of the measured plasma and platelet analytes; and the estimated inter-individual variation for each analyte. The highest value in each column is in bold font.

\* $P < .01$ ; <sup>†</sup> $P < .001$ ; <sup>‡</sup> $P < 0.0001$

The measurement of plasma and platelet TFPI, fV, and PS in a relatively large population of healthy individuals allowed for detailed examination of how their concentrations change with age and sex. Females have significantly lower plasma total TFPI, TFPI $\alpha$ , and PS than males. However, these three proteins progressively increase with age in females, but not in males, to the point where they are at similar concentration in men and women over age 70. In contrast, plasma fV did not differ between men and women and only minimally increased with age. A distinctly different correlation pattern was present for platelet proteins. The platelet TFPI concentration does not correlate with the platelet fV or the platelet PS concentration. However, the concentrations of platelet PS and fV strongly correlate. These findings are consistent with PS and fV localizing to platelet  $\alpha$ -granules,<sup>29,30</sup> while TFPI $\alpha$  does not,<sup>10</sup> and strongly suggests that TFPI $\alpha$  is not bound to either PS or fV within quiescent platelets. The sex and age specific differences of the platelet pools of TFPI $\alpha$ , fV and PS did not mirror those of the plasma pools. TFPI $\alpha$  only slightly increased with age in both sexes, and platelet concentrations of fV or PS remained stable with increasing age. The only platelet protein to differ between the sexes was fV, which was slightly higher in females than males.

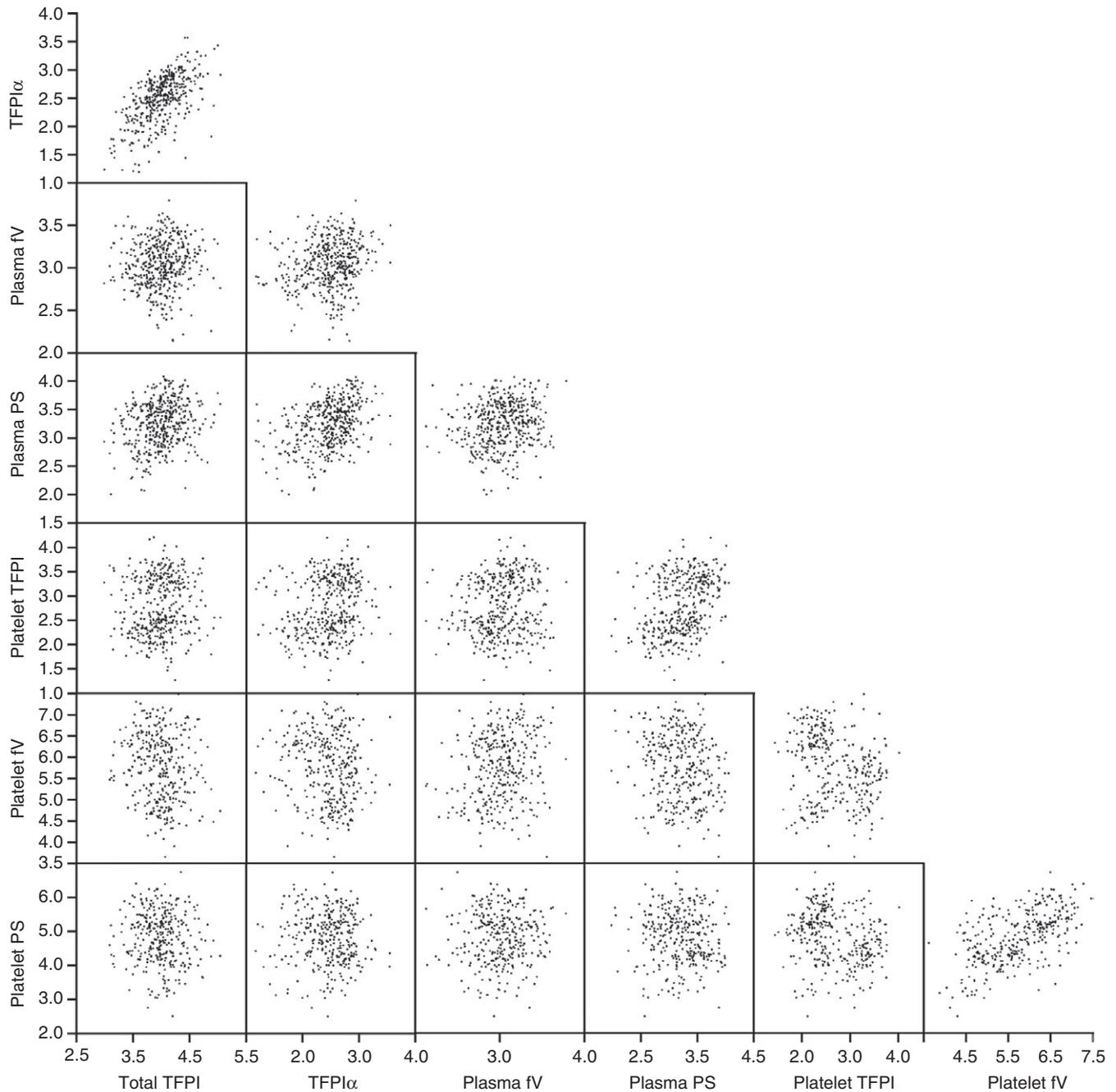
The disparate associations between sex and age, and the determined plasma and platelet protein concentrations, suggest that endogenous hormonal differences between pre- and post-menopausal women have a greater impact on the plasma concentrations of TFPI $\alpha$  and PS than on their platelet concentrations. In our study population, the influence of exogenous estrogens was similar. The use of oral contraceptives or estrogen replacement therapy was not associated with altered plasma or platelet fV. Conversely, these therapies were associated with reduced plasma TFPI $\alpha$  and total TFPI, but did not influence platelet TFPI $\alpha$ , in agreement with the recent findings of Winckers and colleagues.<sup>36</sup> Finally, these therapies were associated with reduced platelet PS, but not plasma PS. The latter is somewhat surprising, given that others have reported decreased plasma total PS concentrations in response to exogenous estrogen therapy.<sup>37</sup> This is observed only in response to oral contraceptives and not hormone replacement therapy. Individuals on either therapy were analysed as a single cohort in the

current study, and therefore it could be expected that an influence of oral contraceptives on plasma total Protein S might not be observed.

Possibilities for the effect of estrogen on both plasma TFPI and PS have previously been suggested as coupled secretion of TFPI and PS from the endothelium; protection of TFPI from proteolytic degradation by binding PS<sup>15</sup>; or common transcriptional regulation of TFPI and PS expression.<sup>38,39</sup> Given that PS is stored in platelet alpha granules while TFPI is not, it is likely that synthesis and storage of the two are not linked in platelets. The differences in production of the two proteins by endothelial cells and megakaryocytes suggest that there is cell-specific regulation of TFPI production either at a transcriptional level through differential expression of estrogen response elements or by differential expression of estrogen receptors. Further research is needed to elucidate the biochemical mechanism underlying the estrogen-dependent differences between plasma and platelet TFPI and PS expression.

Plasma TFPI and PS are produced by endothelial cells.<sup>34,40</sup> Evidence suggests that platelet TFPI and PS originate from endogenous production in megakaryocytes.<sup>10,41</sup> Platelet PS has been hypothesized to be absorbed from plasma, however, the data presented here, showing a lack of correlation between plasma and platelet PS, suggests that this is not the case. Conversely, platelet fV, originating from plasma, is endocytosed by megakaryocytes and stored in platelet  $\alpha$ -granules.<sup>42,43</sup> Accordingly, a weak positive correlation ( $r^2 = .19$ ) between plasma and platelet fV was observed.

Plasma and platelet TFPI, fV and PS expression vary with race. For example, plasma TFPI $\alpha$  and PS concentrations are higher in Caucasians than African Americans, and Caucasians have higher platelet TFPI and lower platelet fV and PS than African Americans. The lack of platelet TFPI has been shown to increase intravascular thrombus size following vascular injury in a murine model.<sup>23</sup> Thus, one could speculate that the decreased platelet TFPI in African Americans may contribute to their increased risk of myocardial infarction and stroke.<sup>44</sup> Although clear inter-racial variations were identified, the inter-individual variation of plasma total TFPI, TFPI $\alpha$ , and fV, and platelet fV and PS was much larger. In plasma, each protein concentration varied by



**FIGURE 3** Scatterplot matrix of measured analytes. Shown is the logarithm of values obtained for all analytes measured, plotted against each other. Significant correlations between plasma total TFPI and plasma TFPI $\alpha$  ( $r^2 = .60$ ), platelet factor V and platelet Protein S ( $r^2 = .58$ ), and plasma TFPI $\alpha$  and plasma Protein S ( $r^2 = .40$ ) were observed. Weaker correlations were observed between other analytes (see Table S5 for a complete list). Concentrations of each protein are presented in Figures 1 and 2

approximately 5- to 10-fold, which is consistent with previous studies.<sup>45,46</sup> In platelets, an even larger concentration range was observed, at approximately 10-fold for TFPI, and approximately 20-fold for fV and PS. This suggests that the effect of race on the concentration of these proteins is minor relative to random variation. For both plasma Protein S and platelet TFPI, the racial differences observed were greater than the estimated inter-individual variation, suggesting that these differences cannot be explained by inter-individual variation alone. Further studies are required to elucidate the underlying

mechanisms resulting in the racial differences observed with these proteins.

In summary, TFPI, fV, and PS are present in plasma and platelet pools where they interact to modulate intravascular pro- and anti-coagulant responses. The plasma forms of these proteins have been well studied over many years, while studies of the platelet forms are more recent. The data presented here reveal that the plasma and platelet pools of these proteins behave independently in regards to demographic expression patterns, response to estrogen therapies and their co-localization with

each other. These findings suggest that the plasma and platelet pools of these proteins may differentially modulate hemostatic and thrombotic responses.

## AUTHOR CONTRIBUTIONS

P.E.R Ellery designed and performed experiments, interpreted data and wrote the manuscript. I. Hilden designed experiments, interpreted data and wrote the manuscript. K. Sejling performed statistical analyses, interpreted results and wrote the manuscript. M. Loftager designed experiments and interpreted data. N.D. Martinez performed experiments and interpreted data. S.A. Maroney designed and performed experiments and interpreted data. A.E. Mast designed experiments, interpreted data and wrote the manuscript.

## RELATIONSHIP DISCLOSURE

A.E. Mast receives research grant funding from Novo Nordisk. I. Hilden, K. Sejling and M. Loftager are employees of Novo Nordisk.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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